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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John C. COX *et al.*

Title: IMMUNOGENIC COMPLEXES AND METHODS RELATING THERETO

Appl. No.: 09/506,011

Filing Date: February 17, 2000

Examiner: S. Foley

Art Unit: 1648

## DECLARATION UNDER 37 CFR 1.132

Commissioner of Patents
Washington, D.C. 20231

Sir:

I, John Cox, declare that:

1. I am a citizen of Australia. A copy of my curriculum vitae is attached.

2. I am an Intellectual Property Consultant. A major client for these activities is CSL, the assignee of U.S. application serial No. 09/506,011 ("the applicant"), and I am a coinventor of subject matter claimed therein.

3. I have reviewed an Office Action for the application, mailed February 13, 2002, and the prior art cited therein. The Office Action states that Nakanishi anticipates or, in the alternative, renders obvious, claims 1-8 and 12-14 of immunogenic complexes.

4. The examiner takes the position that Nakanishi made negatively-charged vesicles, all of which contain phosphatidylcholine and have been enhanced to become more negatively charged by the addition of phosphatidic acid. While the examiner admits that Nakanishi does not explicitly teach that the antigens have a positive charge and are electrostatically associated with the negatively-charged vesicle, she urges that both of these features would have been inherent, because "the negatively charged end of [phosphatidylcholine] would be found in the interior of the vesicle, which associates directly with the protein antigen." She further alleges that there would be an electrostatic interaction

between the negatively-charged phosphatidylcholine of the vesicles and the antigen "since some amino acids inherently have a positive charge, and would be naturally attracted to the negative charge of the phosphatidylcholine, creating an electrostatic association between the antigen and the vesicle in some degree.".

5. A careful reading of Nakanishi, however, reveals the flaws in this reasoning. In the first instance, the conclusion that "the negatively charged end of [phosphatidylcholine] would be found in the interior of the vesicle" evidences a misunderstanding of phospholipid, and hence liposome, structure. The examiner was correct in the Action dated 23 May 2001, when she stated that phosphatidylcholine is an amphipathic molecule. The allegation that a negatively-charged end of this molecule is found in the interior of a liposome is incorrect, however. Liposomes consist of cholesterol, and one or more phospholipids. In their simplest manifestation, they are a bilayer membrane with the hydrophilic headgroup exposed at the internal and external surface. Each layer of the bilayer is aligned so that the hydrophobic ends meet at the middle of the bilayer and the hydrophilic ends are on the surface. A phospholipid consists of two hydrophobic chains, a phosphate group and a head group. The phosphate group carries a negative charge, while the head group may be positively-charged or neutral. Phosphatidylcholine (PC) has *both* a positive (choline) and negative (phosphate) entity exposed at the internal and external surfaces of the membrane, resulting in an overall neutral charge, *i.e.*, the liposome will be neutral at *both* its internal and external surface. Phosphatidic acid (PA) lacks a head group and so is overall negative. In either case, the hydrophobic chains point toward the hydrophobic side of each layer, and the phosphate and head group point toward the hydrophilic surface of the layer. The attached page from Lehninger "Biochemistry" 1970 (p. 785) illustrates formation of a bilayer membrane. By convention, the solid circles are the charged headgroups and the wavy lines the fatty acid chains.

6. Nakanishi used conventional techniques to combine antigen and liposomes. According to such techniques, loading of liposomes is achieved by high pressure agitation, by vortexing or otherwise mixing a lipid film with an aqueous solution of antigen. The loading is very inefficient, as shown by the fact that the external aqueous volume after this process is vastly greater than the encapsulated aqueous volume. No worker in the field prior to the present invention suggested that this inefficiency could be overcome by selecting lipids for preparing liposomes which had a charge suitable for binding the antigen of choice to be loaded.

7. There is no basis for assuming any sort of electrostatic interaction in Nakanishi. Nakanishi uses only two antigens, chicken egg albumin (OVA) and beta-galactosidase. Chicken egg albumin has a pI of 4.9, and beta-galactosidase has a pI of 4.6. Assuming that the antigen and MLV are co-dispersed at 7.6 (the pH stated for the control), both antigens would be negatively charged in Nakanishi. Nakanishi purports to make combinations of these two negatively-charged antigens with negatively-charged, positively-charged and neutral liposomes. This clearly shows that the intention in Nakanishi is not to create an electrostatic interaction. Moreover, as noted above, Nakanishi follows conventional techniques, according to which a relatively inefficient loading of a liposome is achieved by vortexing or otherwise mixing a lipid film with an aqueous solution of antigen.

8. Nakanishi focuses on the interaction between liposome and macrophages, ***not*** the interaction between liposomes and antigen. Nakanishi in Figure 4 shows that all liposomes, regardless of charge, are able to enhance antibody response. Conversely, Nakanishi shows in Figure 2 and repeatedly states in the text that only positively-charged liposomes are able to induce CTL responses. This was attributed to the fact that only positively-charged liposomes were able effectively to bind to the surface of murine macrophages. Nakanishi does not teach that charged liposomes can be combined with oppositely-charged antigens to improve association between the two, and specifically teaches that only positively-charged liposomes are able to induce CTL responses.

9. The examiner also alleges that the specification does not support vaccine claims that are broad enough to encompass "any positively charged protein and any negatively charged adjuvant." At page 2 of the Official Action, in particular, the examiner states that the application, while "enabling for peptides HpC, SYI, YPH or RPQ with six lysines (K (H) associated with cardiolipin (CDL) or diphosphoryl lipid A (DPL), or CHL, does not reasonably provide enablement for any positively charged protein and any negatively charged adjuvant...."

10. At the outset, I note that HpC is a protein antigen and not a "peptide", with the examiner's meaning. Furthermore, the association was shown to occur when the negative charge on the charged organic complex was increased by incorporation of DPL, an aspect unrelated to whether or not "six lysines" are present. Indeed, the exemplified protein did not contain a six-lysine addition.

11. More particularly, the examiner urges that "a specific CTL response directed against the antigen has not been demonstrated. Example 4 on page 25 that applicant has referred to demonstrates that a CTL response was generated in response to ESO

associated with ISCOMATRIX™, but there was no CTL response generated against ESO alone. Since there is no data provided for whether a CTL response is generated against the adjuvant alone, it cannot be determined whether the CTL response observed is directed against the adjuvant alone or the antigen associated with the adjuvant."

As the examiner will appreciate, the immune system provides two highly sophisticated and distinct mechanisms for recognising and clearing foreign antigen in a *specific* manner, these mechanisms being humoral and cellular immune responses. The cellular immune response can be further categorized in terms of the helper T cell response and the cytotoxic T cell response. As its name suggests, the cytotoxic T cell response is predicated on the cytotoxic nature of the effector mechanism which these cells utilise.

In terms of the means by which antigen recognition is achieved, the B cells of the humoral immune response express an antibody which binds directly to an epitopic region of the native form of an antigen. However, such direct binding does not occur in the context of a cytotoxic T cell response. Rather, the antigen binding molecule of the T cell, being the T cell receptor, will only bind to an isolated amino acid *peptide* derived from the antigen in issue, which peptide is presented to the T cell receptor as a complex together with a major histocompatibility class I ("MHCI") molecule. Subsequently to B or T cell receptor binding, stimulation of the B or T cell is achieved where the appropriate co-stimulatory signals are provided to the cell. Stimulation of cytotoxic T cells to respond to a specific foreign antigen thereby relies on the occurrence of the significantly more complex process of protein antigen degradation and re-expression of the peptides derived thereby in the cleft of the MHCI molecule which is anchored to the cell surface of an individual's cells. This process is termed "antigen processing". Briefly, when a protein or polypeptide is introduced into the cytosol of a cell protein, degradation is effected by the proteasome, a multi-catalytic protease complex which specialises in processing *proteins* to peptides. The subsequently generated peptides are actively transported from the cytosol to the lumen of the endoplasmic reticulum where some of the peptides bind to the cleft region of an MHCI molecule, which MHCI-peptide complex is then delivered and anchored to the cell surface. Accordingly, the MHC molecule cannot bind the antigen in its native form. Since the cleft is of a defined size, due to it being closed at both ends, only *peptides* of about *9 amino acids* can be accommodated.

Once the MHCI-peptide complex has been expressed on the cell surface, it is available to be bound by a T cell receptor which specifically recognizes the bimolecular complex of the specific form of MHCI and the peptide itself. If the T cell receptor does not recognize both the specific form of the MHCI "presenting" the peptide and the peptide itself,

T cell receptor binding will not occur. This co-recognition of MHCI and antigen is termed "MHC restriction". It should be noted, however, that binding of the T cell receptor to this bimolecular complex will, of itself, be insufficient to stimulate activation of the T cell to which the T cell receptor is anchored. As occurs in the context of B cell stimulation, appropriate co-stimulatory signals, such as binding of the CD8 accessory molecule to the side of the MHCI molecule, are also required.

It should be noted, however, that although all the cells of the body express MHCI/peptide complexes, it is largely the antigen presenting cell subgroup of cells which can also provide the necessary co-stimulatory signal to induce a new cytotoxic T lymphocyte response. Still further, there is diversity among the MHCI molecules which are expressed by any given individual. Specifically, there are more than 40 separate alleles of the MHC gene. Any given individual will express a subgroup of these alleles, meaning that any given individual will express on the surface of a single cell a range of MHCI polymorphic forms, but not necessarily all of the forms which are found in the population. This has significance in that the particular form of any given MHCI molecule will determine which peptides can bind into the cleft of that MHCI molecule. That is, any given polymorphic form of MHCI will have the capacity to bind to a range of peptides, but not all peptides. It is for this reason that the expression of a range of polymorphic MHCI forms is necessitated such that a capacity to present to the T cell as many peptide forms as possible is facilitated.

Accordingly, the specificity of a cytotoxic T lymphocyte response is dependent on two factors:

(i) The primary amino acid sequence of the antigen, which amino acid sequence determines the sequences of the individual peptides which are generated subsequent to the actions of the proteasome; and

(ii) The genetic background of the individual which determines which polymorphic forms of MHCI are expressed by that individual and thereby determines the range of peptides which can be bound by those MHCI molecules and presented to that individual's cytotoxic T Cells.

Any given T cell receptor recognizes a combination of a specific antigen derived peptide sequence and a specific MHCI polymorphic form. The T cell receptor will not bind to an MHC-peptide complex if either one of these components is of the incorrect form. In the absence of correct T cell receptor/MHC-peptide interaction, activation of the T cell to which the T cell receptor is anchored cannot occur. *In vitro* assays which are utilised to screen for a cytotoxic T cell response to a specific peptide thereby require co-culture of a

cytotoxic T cell population which express a receptor recognizing the peptide in issue as complexed to the MHCI molecule of a given target cell. Binding of the T cell receptor to this peptide-MHC complex will be indicative of cytotoxic T cell recognition while, in the more reliable assays, killing of the target cell will further indicate the successful stimulation of the subject T cell and induction of its cytotoxic activity.

A review of the examples of the specification reveals that the documented CTL responses were generated specifically against the protein or peptide employed as a component of the immunizing formulation. At page 25, lines 8-15, for instance, the specification indicates that splenocytes harvested from the immunized mice were re-stimulated with EL4 HHD cells that had been sensitized with ESO peptide and irradiated. These cells were cultured and then used as effectors in a standard 51Cr-release assay against EL4 HHD cells sensitized as for re-stimulation. Similarly, each of Examples 6, 9, 14 and 17 detail cytotoxic T lymphocyte-response assays that were measured specifically against the immunizing protein or peptide. Thus, ***these examples provide every indication that the response generated by these mice was directed specifically to a peptide region of the immunizing antigen***. In other words, these *in vitro* assays unequivocally demonstrate the generation of a CTL response to a peptide region of the protein with which the mouse was immunized.

The CTL response cannot be directed to the Iscomatrix adjuvant because Iscomatrix, by definition, consists of the non-protein molecules saponin, cholesterol and lipid (generally but not necessarily phospholipid). It *does not* contain protein or peptide. A CTL response can only be generated to a molecule which is capable of undergoing proteosome degradation and presentation in the cleft of an MHCI molecule. It is thus not possible for a CTL response to be directed to Iscomatrix or to any of the components of Iscomatrix, being non-protein molecules.

The appropriate control for *in vitro* CTL studies is the use of target cells lacking presentation of the specific peptide in the context of their cell surface MHCI molecules. Such controls were present in all the CTL studies in the present application and were negative. A positive result, i.e., killing and hence Cr release with cells transfected with the target peptide or protein therefore establishes the specificity of the response to the peptide. Analogous target and control cells (EG7 and EL4 respectively) were used by Nakanishi *et al.* (1997).

12. The examiner further alleges that the specification does not demonstrate that there is any predictability with how well proteins will associate, on the

grounds that TYQ in Example 17 was treated to increase positivity by the addition of 6H and 6K, associated with a negatively charged adjuvant, and yet generated only very weak responses. The recombinant polytope of Example 17 contains six CTL epitopes in tandem. Responses to four of the six peptides were measured and reported. The results showed strong CTL responses to three epitopes and less strong responses to the fourth. It is respectfully submitted that:

(i) The construct used for immunization in this example was the recombinant polytope, not the individual epitopes.

(ii) The positivity of the polytope was increased by addition of the 6K and 6H. Such tags were not applied to the individual epitopes.

(iii) The success of the formulation was measured by determining CTL responses to certain of the component epitopes within the polytope.

(iv) A weak CTL response is nevertheless a positive response.

In summary, the formulation utilized in Example 17 did induce a strong CTL response to three of its component epitopes, and a weaker response to the fourth (TYQ). Thus, the overall CTL response generated to this polytope was a strong response, with relative differences in response observed only when one investigated the extent of response to the individual epitopes within the polytope.

In addition, it is noted that Example 14 describes studies with the equivalent synthetic polytope. Example 14 shows that good CTL responses were induced to peptide TYQ.

13. The examiner urges that undue experimentation is required to practice the invention with antigen/organic complex combinations other than those exemplified. Yet only minimal experimentation of a routine nature is required to practice the invention with combinations other than those reported in the specification. The specification teaches how to select and make a charged organic complex, how to bring about association between a charged organic complex and a charged antigen and how that association can be increased by modifying either or both of the organic complex and antigen. The specification also teaches ways of testing the resulting association, and shows that when association has been achieved, benefits such as CTL response result. Any necessary experimentation is readily carried out by a skilled technician, and is not undue in this field.

14. In addition to the foregoing, attached hereto is a published paper [Polakos *et al.*, 2001] which describes results of studies with the hepatitis C core antigen formulated by the method of the present invention. Separately attached is a report on very

recent studies using a hepatitis C polyprotein which is a fusion of five hepatitis C proteins. It can be seen that each of the fusion components is able to induce a CTL response in macaques although not all fusion components were recognized by each macaque. Similar data has also been obtained in chimpanzees although in this study none of the five chimps responded to core.

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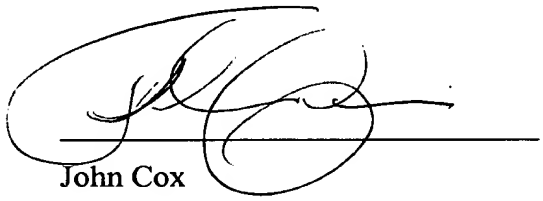
I hereby declare that all the statements made herein of my known knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

28th March 2003
Date



John Cox